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Expression of small, therapeutic RNAs in human cell nuclei

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Effective intracellular expression of small RNA therapeutics depends on a number of factors. The RNA, whether antisense, ribozyme, or RNA aptamer, must be efficiently transcribed, stabilized against rapid degradation, folded correctly, and directed to the part of the cell where it can be most effective. To overcome a number of these problems we have been testing expression cassettes based on the human tRNA^{met} and U6 snRNA promoters, in which transcripts encoding small RNA inserts are protected against attack from the 3′ end. Transient expression in cultured cells results in 10^5–2×10^7 full-length transcripts per cell, depending partially on the promoter construct used but also on the nature of the insert RNA. 5′ γ-Phosphate methylation (capping) depended, as expected, on the inclusion of specific U6 snRNA sequences from positions +19 to +27. In situ localization of the transcripts shows that both tRNA and U6 promoter transcripts give primarily punctate nuclear patterns, and that capping of transcripts is not required for nuclear retention. Several different insert RNAs directed against HIV-1 were tested by cotransfection with HIV-1 provirus and assay for subsequent viral reverse transcriptase production. These include antisense RNA, hairpin and hammerhead ribozymes, and RNA ligands (aptamers) for Tat and Rev RNA binding proteins. Results show that Rev-binding RNAs efficiently block HIV-1 gene expression, whereas other RNAs have little or no effect when expressed in these cassettes.

Keywords: *aptamers; HIV-1; ribozymes; small nuclear RNA; U6*

Introduction

A number of different strategies have been envisioned for inhibiting viral infections and uncontrolled cell growth by delivering therapeutic nucleic acids to affected cells. In approaches where the cells are induced to synthesize their own protective nucleic acids from exogenously supplied genes, the choice of nucleic acid is RNA because the cell can produce intracellular single stranded RNA in quantity. Several types of RNA inhibitors are theoretically possible, including antisense RNA, catalytic RNA (ribozymes), and high-affinity RNA ligands, termed 'aptamers' or 'decoys'.[1–11]

To deliver successfully the RNA inhibitors to their intended targets in the appropriate subcellular compartments it is necessary first to identify promoter strategies and RNA signals that specify the desired localization. A specific and promising example of this has been provided by embedding a ribozyme in a pseudoviral transcript that might be copackaged with the *bona fide* retroviral transcripts, thus providing directed access to the target sequence.[12] Many types of inhibitors are likely to require either nuclear or less constrained cytoplasmic delivery, however. In principle this should be possible for many targets, since RNA signals for localization to the nucleus, nucleolus, and cytoplasm have been identified.

U6 is a small, stable RNA that exists as an abundant nuclear ribonucleoprotein (U6 snRNP) in all human cells, where it plays central roles in both spliceosome assembly and catalysis in nuclear pre-mRNA splicing. All of the major transcriptional promoter elements for RNA polymerase III (pol III) are upstream of the transcription start.[13–16] This upstream U6 promoter strategy confers a potential advantage over the use of other pol III promoters (5S rRNA and tRNA classes), where insert RNA must be expressed as a fusion with the RNA sequences encoding the intragenic promoters. This is of special concern in strategies using viral RNAs, which might have cytotoxic effects, for example Refs 17 and 18). A number of such tRNA gene fusions have been tested both in experimental organisms and as potential expression cassettes for small RNAs in gene therapy.[10,11,19–21] An additional advantage of the U6 gene is that individual genes are heavily expressed in human cells. Transcription of only a few copies of the U6 gene (out of 200 or more pseudogenes[22]) results in roughly 400 000 copies of RNA per cell.[23] The U6 primary transcript is normally trapped in the nucleus, appearing both in the nucleoplasm and in localized nuclear 'speckles'.[24] This is in contrast to other small nuclear RNAs synthesized by pol II, which exit to the cytoplasm and need to acquire an extensive protein complex bound to internal RNA sequences for re-entry into the nucleus.[25,26]

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In this report the human U6 promoter and different amounts of U6 RNA coding sequence have been used to drive the expression of small ribozymes, antisense oligoribonucleotides, and RNA aptamers directed against HIV-1 RNA and proteins. The amount, degree of processing, and location of the RNA expressed in human cells is examined, as is the degree to which expression of these RNAs inhibits HIV-1 gene expression.

Results

Promoter cassettes and inserts

Figure 1a shows a schematic representation of the U6 gene cassette that was used for expression of small RNA inserts, with the expected secondary structure of the transcripts below. Figure 1b shows similar information for a tRNA gene cassette used for comparison (modeled after those used previously).[10,11] All of the transcripts tested here have strong, artificial stems with stable tetraloops after the insert sites and immediately preceding the polyU terminator for pol III. This structure is expected to protect against 3′–5′ exonuclease attack, the most prevalent small RNA breakdown pathway, and to reduce the chances of the 3′ trailer interfering with the insert RNA folding. The restriction cloning sites between the inserts and the flanking sequences (5′ leaders and 3′ stems) serve as structural spacers.

The tRNA transcript contains only the leading three quarters of the native tRNA sequence. It was expected that this structural perturbation would prevent pre-tRNA 5′ and 3′ end processing, thus keeping the 5′ end of the insert protected and preventing export from the nucleus. The U6 expression cassettes were of three types. All had the human U6 gene upstream sequence[14,15] from positions −1 to −265 and the same 3′ stem at the transcript

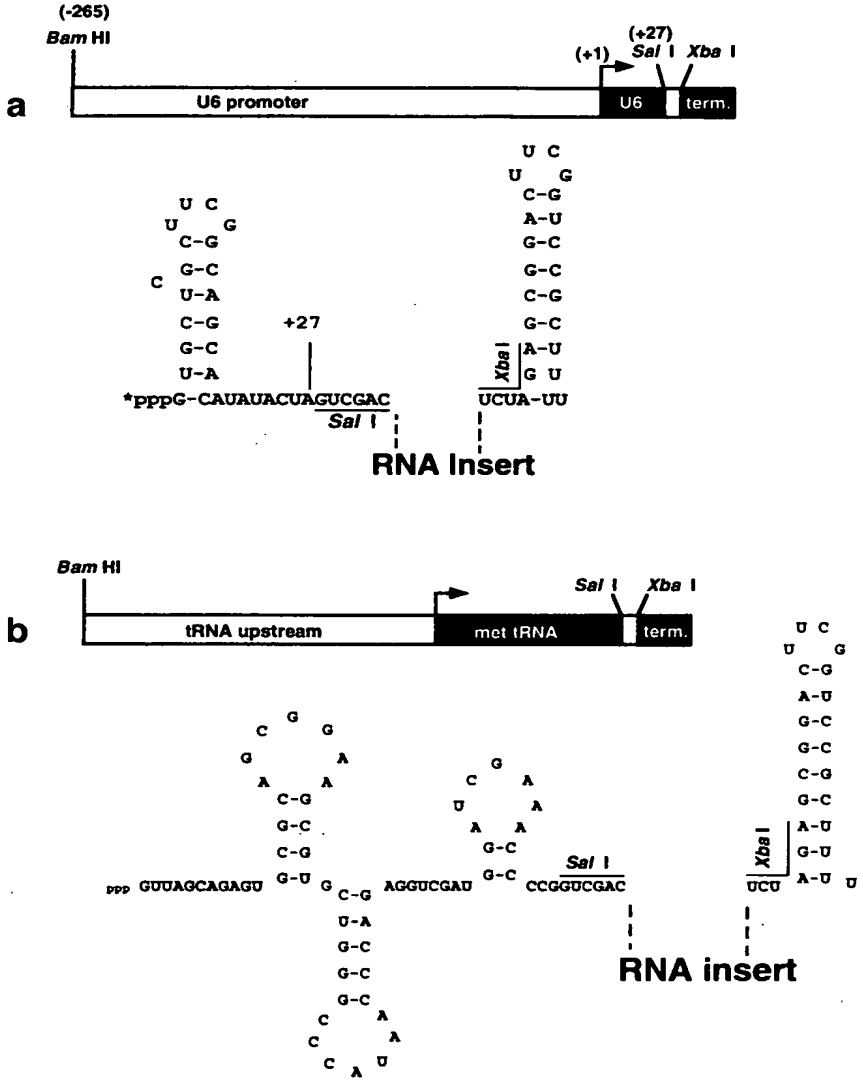

Figure 1 U6 snRNA and tRNA promoter expression cassettes. Schematic representations of the upstream (open boxes) and transcript portions of the U6 and tRNA expression cassettes are shown, along with the sequences and predicted structures of the expected primary transcripts.

terminator. The only difference was that the three types of transcripts began with different amounts of U6 RNA. 'U6+1' began with +1 as the *Sal*I cloning site for the insert, thus having no U6 RNA and only a short leader corresponding to the *Sal*I sequence. 'U6+19' had the *Sal*I site inserted after +19, which includes the leading stem of U6. 'U6+27' had the *Sal*I site after +27. This construct, shown in Figure 1a, also includes the full sequence required for 5' γ-phosphomethyl 'capping' and stabilization.[16,27–29] The +19 to +27 sequence was implicated in stability, as well as capping, and the capping was hypothesized to signal nuclear retention. It was possible on the basis of the existing data, however, that only the +1 to +19 stem was required for stability and that this RNA would find its way to the cytoplasm without the 5'-γ-phosphomethyl cap. In practice, steady-state levels of RNA increased from U6+1 to U6+19 to U6+27, but the localization remained similar (see below).

Six general types of inserts were cloned into these cassettes and tested for the levels of RNA expression and their degree of processing. Four of the inserts are shown in Figure 2. Two of these are ribozymes, either the hammerhead or hairpin directed against the same site in the U5 region successfully targeted previously.[30,31] For the hammerhead, two lengths of annealing domain were used (HH1 *versus* HH2) to test either aggressive annealing or better product release to encourage turnover. In addition, a simple antisense clone was produced corresponding to the same 28 nt annealing sequence as the longer hammerhead, but without a catalytic domain. Two types of aptamers were initially tested. One is minimal HIV-1 TAR (in the 5' leader LTR transcript) that is required for Tat binding, and the other is an aptamer corresponding to the small Rev-binding element (RBE) subdomain of the RRE (at the end of the *env* gene).[32] Additional aptamers of varying sequences that were previously selected for high affinity to Rev were also tested for effects on HIV-1 expression (see below, 'Effectiveness in preventing HIV-1 proviral gene expression').[33]

Expression of RNA inserts

Figure 3 shows Northern blot analysis of the steady state accumulation of RNA from the different promoter/insert strategies, 2 days after transfection into human 293 cells. For each construct, the lanes were probed with a labeled DNA oligonucleotide complementary to only the unique RNA insert. The only significant accumulation seen in cells from any of our constructs is the nearly full-length form, suggesting that if any processing occurs to remove the 5' leaders or 3' stem, the rest of the transcript does not survive long. The sizes of the RNAs are consistent with a series of RNAs that are 1–10 nucleotides shorter than the expected primary transcripts, which terminate at the fourth U in the terminator stem (Figure 1). This would be consistent with slow exonuclease attack on the 3' terminator stem.

Quantitative assessment of the level of RNA produced per cell in the transient transfections was obtained using Phosporimager counting of the major bands with normalization to both the percentage transfection efficiency (usually about 70%, estimated by cotransfections with the β-galactosidase gene) and the endogenous level of U6 snRNA on the same blot (shown in the lower panel). The approximate steady state levels of RNAs from the various constructs is shown in Table 1, assuming 400 000 copies of endogenous U6 snRNA per cell.[23] We also tested RNA from a limited number of constructs for the presence of a 5' γ-phosphomethyl cap by Northern analysis after immunoprecipitation with antibodies directed against this cap.[27] The results are summarized in the right hand column in Table 1. The signal, after normalization for the efficiency of endogenous U6 snRNA precipitation, showed essentially the expected results for capping. The U6+27 transcripts capped with reasonable efficiency, while the U6+1 RNAs and tRNA fusions were uncapped and the U6+19 capped poorly (8%).

The levels of RNA from different promoters with the same insert were essentially what was expected. tRNA-fused RNA levels were high, as were the U6+27 transcripts that were predicted to be most stable because of the cap. The U6+19 and U6+1 transcripts had progressively lower levels within each insert group. The levels of RNA in response to the insert type changed even more dramatically, however, and in ways that were not predictable in advance. The antisense inserts, which we would have predicted to be the most open to endonucleolytic attack because they lack strong structure, instead accumulated to the highest level of any tested insert. The hairpin ribozyme, TAR structure, and RBE were maintained at slightly lower but still high levels. In contrast, the hammerhead ribozyme levels were 15- to 20-fold lower than the levels of hairpin in the same promoter backbones.

Destinations of expressed RNAs

In situ hybridization methods were used to locate transcripts with probes specific to the inserts. Digoxigenin-labeled DNA probes for U6 snRNA or the insert plus terminator stem were annealed to the RNA in fixed cells, and detected by confocal fluorescent microscopy using FITC antibodies against the digoxigenin-11-dUMP tag. Figure 4a shows a control experiment in which this type of probe was directed toward endogenous U6 snRNA (left panel) and compared to staining for total cellular RNA and DNA with propidium iodide (right panel, DNA stains more brightly than RNA). Splicing components like U6 snRNPs are expected to be almost exclusively nuclear, although there is disagreement as to whether the U6 localizes to a large or small number of punctate loci outside of the dark nucleoli (two to four dark spots inside the large nuclei in each panel are nucleoli). In contrast to the one other report of U6 localization,[24] we find the U6 in a large number of punctate nuclear loci reminiscent of other splicing component distribution observed by these fixation methods.[34] It is interesting to note that in the two cells undergoing chromosome condensation and division (lower left and upper right corners of panel), the U6 signal becomes dispersed (non-punctate) throughout the cell. This suggests that when the nuclear envelope breaks down during division, the U6 may lose precise localization and have to be reorganized as the nucleus is reformed.

Insert RNA signal from both tRNA fusions and the U6 promoters localize primarily to the nuclei in the frames shown in Figure 4b. Not all cells stain for the insert RNA and some cells stain more brightly than others, unlike the case for endogenous U6. We attribute the uneven signal to unequal transfection efficiency among cells. The signals for the hairpin ribozyme insert from U6+1, U6+19, U6+27, and tRNA promoters are shown for comparison,

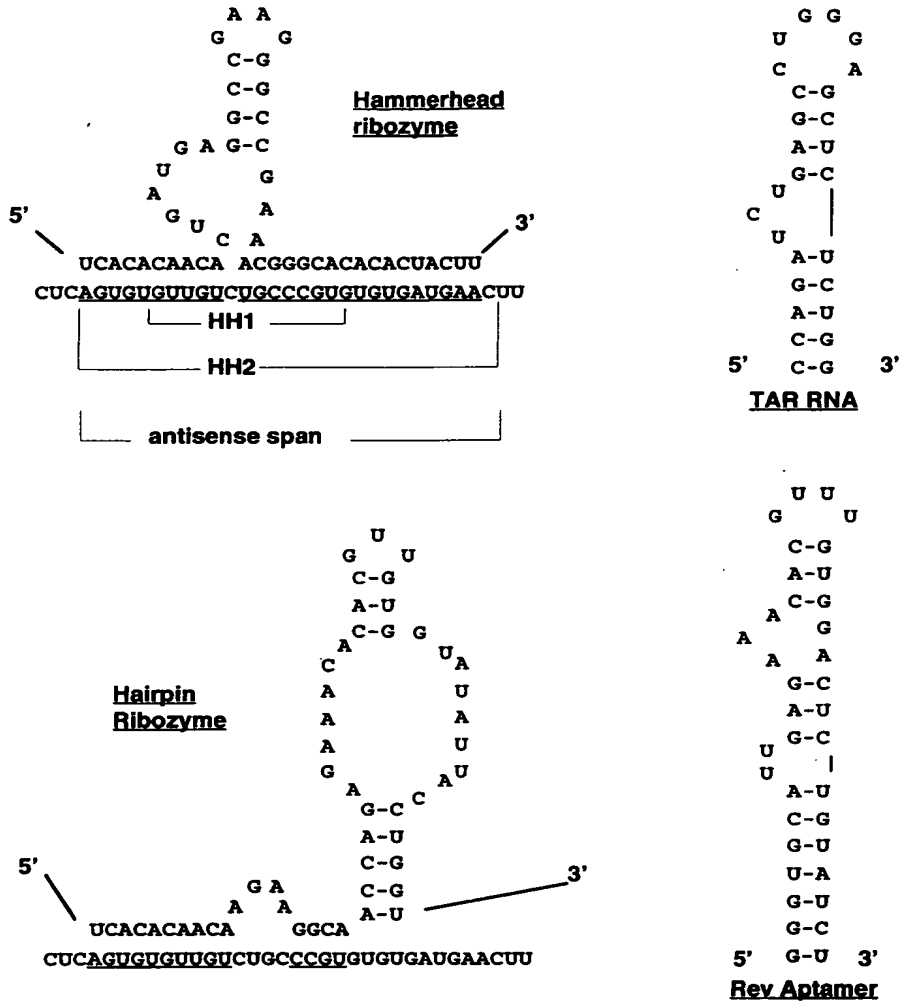

Figure 2 Ribozyme and RNA ligand (aptamer) inserts. The sequences and predicted structures of the RNAs tested for anti-HIV-1 expression are shown. Two hammerhead ribozymes are represented, HH1 with a 12 bp annealing domain directed against the U5 region of HIV-1, and HH2 with a 28 bp annealing domain directed against the same region. The antisense construct used in these experiments (not shown) anneals to the same site in the 5' end of the transcript as HH2. The sequences of the minimal Tat-binding region of HIV-1 TAR RNA and the Rev-binding aptamer RNA are discussed in the text.

with exposure times held constant (Figure 5b). The signal from even the most poorly expressed cassette, U6+1, is easily detected. The signal from different inserts (TAR and RBE) in the U6+27 cassette was very similar to that of the hairpin. It was expected that the unprocessed partial tRNA fusion transcripts would remain nuclear, since previous work in yeast and *Xenopus* has demonstrated that transport out of the nucleus is linked to terminal processing and tRNA splicing. For the U6 transcripts, it is clear that they are primarily nuclear even without the U6 capping signal, or any U6 sequences at all (+1 construct). As the level of the signal increases, the pattern is one of underlying punctate staining with increasingly bright nucleoplasm. In U6+1hp, the signal is primarily punctate, whereas in U6+19 at the same visual settings there is bright, diffuse nucleoplasmic staining overlaying the punctate pattern. There is even some cytoplasmic staining in the brightest cells transfected with tRNA and U6+27 promoters. It is possible that this increasingly diffuse staining reflects saturation of whatever was causing the RNA to locate to the nuclear 'speckles'. The nuclear localization of all of these transcripts, but especially the U6+1 transcript, suggests that nuclear retention might be the default pathway for transcripts that are not specifically directed to the nucleolus or cytoplasm.

Effectiveness in preventing HIV-1 proviral gene expression

Each of the expression constructs was tested for the ability to inhibit HIV-1 gene expression. Cotransfection of the plasmids with pNL4–3 provirus (40:1 ratio) was followed by assay for viral reverse transcriptase (RTase) after 1–5 days. In repeated experiments, day 3 was found optimum in terms of RTase levels and % inhibition and the data shown in Figure 3 are for that interval. The

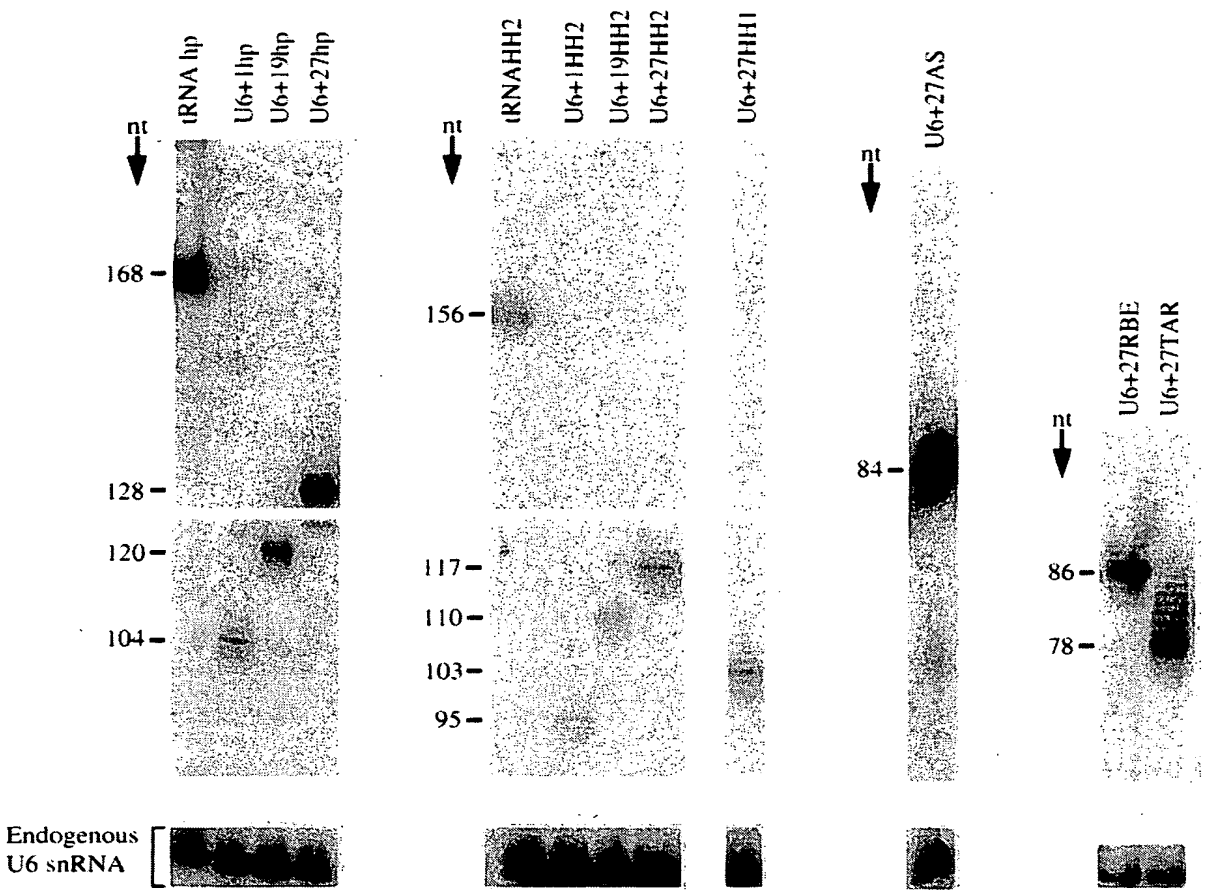

Figure 3 RNA blot analysis of RNAs expressed in transfected cells. RNA was extracted from cells transfected with the indicated plasmid constructs, subjected to denaturing polyacrylamide gel electrophoresis, electroblotted to nylon membranes, and probed with oligonucleotides that annealed specifically to the indicated insert RNAs in individual panels. The signal from probing the same blots was also probed with an oligo complementary to endogenous U6 RNA to provide an internal quantification standard. The estimated sizes of the RNA transcripts, indicated to the left of each panel, were obtained in comparison to in vitro RNA transcripts of 158, 129, 115, 77 and 38 nucleotides run on the same gels.

results were averaged for triplicate assays in two to four independent experiments and are shown in Figure 5. RTase activity is expressed as a percentage of the noninhibited proviral transfection control.

In general these antisense and ribozyme constructs in these particular vectors are not very effective inhibitors (Figure 5a). The HH2 hammerheads with long annealing domains (expressed at the lowest level) are the best of the ribozyme inhibitors, although this is only 50–60% RTase reduction, not much greater than the inhibition attained with higher levels of the antisense to the same region. Similarly, the minimal TAR sequence from HIV-1 was not inhibitory in these vectors. It was only when we inserted an optimized Rev-binding aptamer ('U6+27RBE' in Figure 5a)[32] that good inhibition was consistently obtained. This was shown to be the result of the insert RNA's Rev-binding ability, since several different Rev-binding ligands (Figure 5b),[32,33,35] gave significant inhibition of RTase activity relative to the hammerhead ribozyme insert (U6+27HH control). The four new aptamer sequences all work approximately as well as the original RBE aptamer ('U6+27RBE') when expressed from either the U6+19 or U6+27 cassette (U6+1 was not tried), but less well when expressed from the tRNA cassette. From these results we concluded that high-affinity Rev-binding elements in general inhibit RTase expression from the cotransfection, and that the U6 promoter strategy was working better than the tRNA promoter.

Discussion

There are a number of issues to be considered in developing optimal promoter strategies for delivering small, structured RNAs. Aside from containing signals that specify a preferred destination, the RNAs should be driven to fold in a fashion that both promotes stability and allows inserted RNA folding, independent of the surrounding RNA. Ideally, the therapeutic RNA and any surrounding sequences should not bind cellular factors that are not absolutely essential for function. In such cases high-level expression of the RNA might result in competition for essential proteins and toxicity. At the moment a limited amount is known about RNA signal sequences that determine destinations, but several factors suggest that RNA polymerase III transcription units might be ideal for synthesis and delivery of small RNA

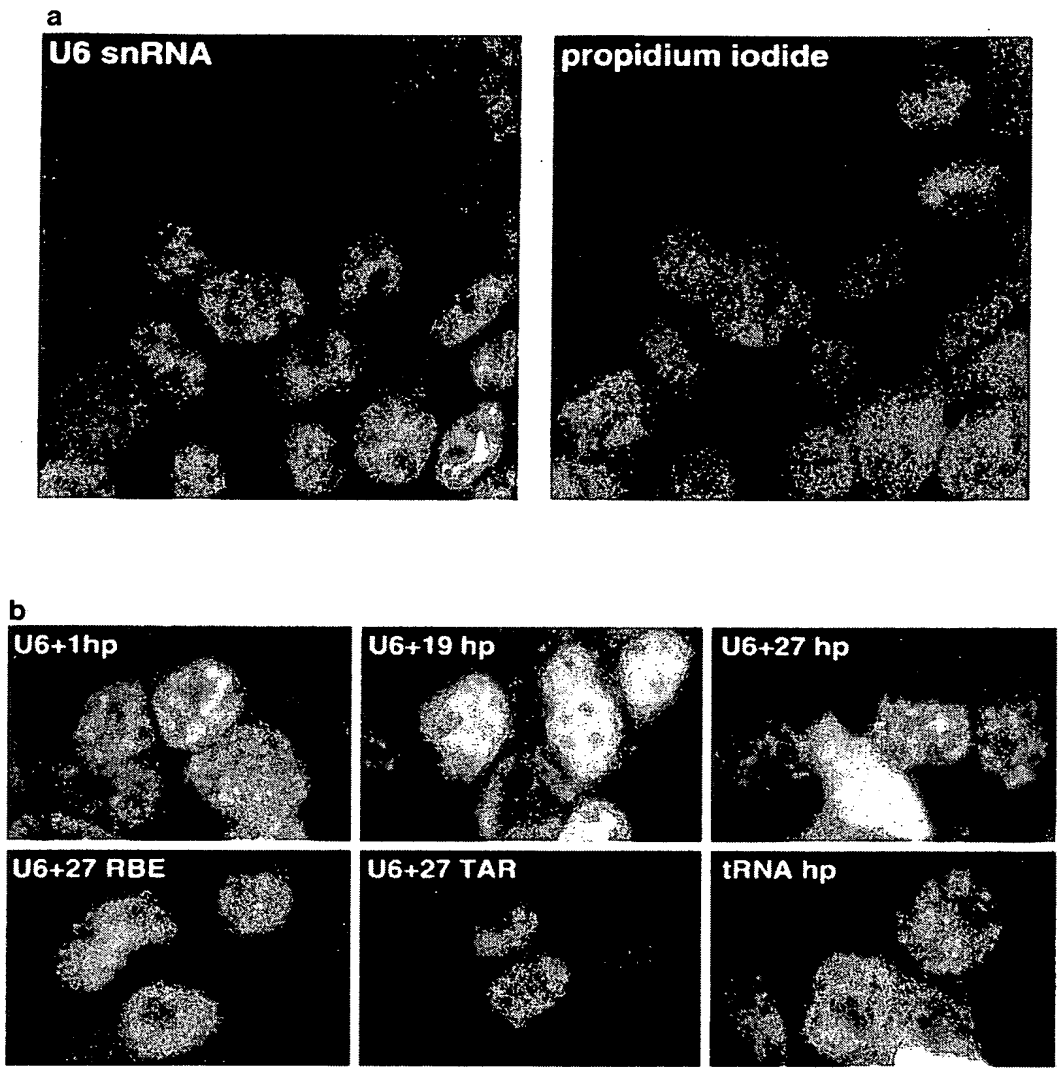

Figure 4 In situ hybridization to endogenous U6 snRNA and expressed RNA inserts. (a) Human 293 cells were probed with digoxigenin-labeled DNA complementary to endogenous U6 snRNA and detected with FITC-conjugated anti-digoxigenin antibodies (left panel) or stained for nucleic acids with propidium iodide. Nuclei and condensed chromosomes stain more intensely with propidium iodide. U6 signal is almost entirely in nuclear speckles in nondividing cells, with dark unstained regions corresponding to nucleoli. In cells with condensed chromosomes, U6 staining is uniformly dispersed throughout the cells, presumably due to nuclear breakdown and loss of normal localization structures. (b) 293 Cells transfected with several different expression cassette–insert combinations were probed for location of the RNA transcripts by the same digoxygenin-DNA–FITC-antibody method used in (a), except that the probe was complementary to the insert RNA and the artificial stem–terminator of the transcripts. Fields are shown for the hairpin ribozyme (hp) synthesized from several U6 promoter variants and the tRNA promoter. Expression of the Rev-binding element (RBE)[32] and the TAR sequence are also shown. Nuclear speckled patterns or staining occurred in all cases, although in cells with particularly heavy staining there appeared also to be diffuse nucleoplasmic and some cytoplasmic staining.

to nuclei. First, these transcription units are normally in the business of synthesizing high levels of small, stable, tightly structured RNAs. In several instances strategies have been devised that allow stabilization and transport of the RNA transcripts while deleting protein binding sites that would restrict their freedom of movement or block exposure of the insert RNAs. We have investigated one of these strategies, the human U6 snRNA promoter, for developing nuclear delivery vehicles for small RNAs.

Because the human U6 promoter is upstream of the transcription start site, transcripts can be expressed that lack all endogenous U6 RNA sequences. We compared this strategy to including either the first 19 nucleotide U6 RNA stem, or including the first 27 nucleotides, thus also providing the sequence known to be required for U6-specific 5′ capping. As expected, including the first 19 and first 27 nucleotides of U6 significantly increased the levels of the RNA transcripts, with the +27 being both capped and increased three-fold over +19. These U6+27 fusions accumulated to roughly the same levels as fusions to partial tRNA 5′ leaders. In all cases accumulated transcripts tended to be near full-length, suggesting that if the terminal structures were effectively blocking exonuclease attack and that if internal cleavages occurred, the resulting RNA did not accumulate.

Table 1 Levels of RNA per transfected cell

Construct	Approximate No. per cell[a]	% γ-Me cap
tRNA-hairpin Rz	780 000	0
U6+1-hairpin Rz	60 000	0
U6+19-hairpin Rz	180 000	8
U6+27-hairpin Rz	470 000	85
tRNA-HH2 hammerhead Rz	22 000	—
U6+1-HH2 hammerhead Rz	2800	—
U6+19-HH2 hammerhead Rz	12 000	—
U6+27-HH2 hammerhead Rz	34 000	—
U6+27-HH1 hammerhead Rz	32 000	—
U6+27-antisense 30 nt RNA	2 200 000	—
U6+27-TAR RNA	1 200 000	68
U6+27-Rev aptamer (RBE)	400 000	78

[a]Normalized to endogenous U6 snRNA, assuming 400 000 U6 RNAs per cell.
RNA levels were obtained by quantification of Northern blot hybridization to ^{32}P-oligo probes specific for the insert RNA ribozyme (Rz), TAR, or RBE. Signal was normalized by hybridization signal of an oligo to endogenous U6 snRNA on the same blot of a denaturing polyacrylamide gel and by estimating the transfection efficiency (±10%) by cotransfected β-galactosidase gene staining. The degree to which the transcripts were capped was estimated in selected cases by immunoprecipitation with anti-γ-phosphomethyl antibody and detection of RNA in the supernatant and bound fraction by quantitative Northern analysis. Precipitation efficiency was normalized to the efficiency of precipitation of endogenous U6 snRNA.

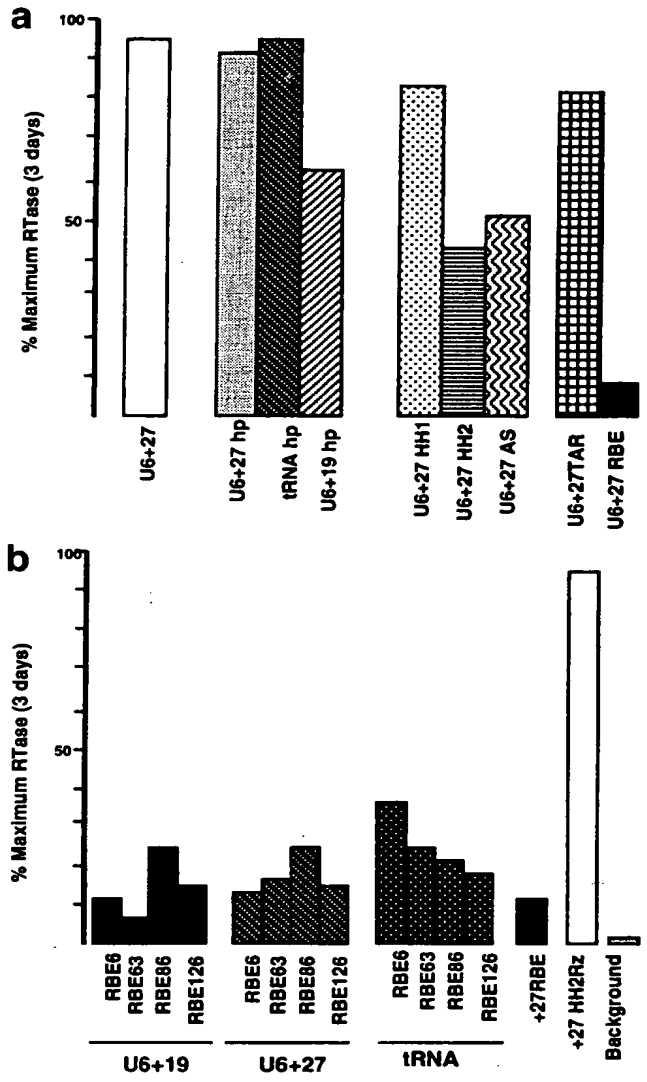

Figure 5 Inhibition of viral gene expression. Plasmid containing the indicated constructs were co-transfected with pNL4–3 HIV-1 provirus into 293 cells with approximately 70% of cells receiving DNA. After 3 days, cell extracts were assayed in triplicate for reverse transcriptase (RTase) activity. Each experiment was repeated two to four times. The values are presented as percentages of the maximum control activities in a given experiment, averaged over the experimental repeats. Effects of the hammerhead ribozymes (HH1 and HH2), hairpin ribozyme (hp), antisense RNA (AS), TAR RNA, and Rev-binding element (RBE)[32] used in the initial studies are shown in Figure 2. The additional RBEs (numbered 6, 63, 86, 126 according to original nomenclature)[33] were derived independently and have different sequences, although they all bind Rev with high affinity.

Two unexpected conclusions regarding the RNA expression were derived from these data. The first is that the exact RNA inserted in the cassettes strongly influences the level of accumulated RNA in the cell, and in ways that are not necessarily predictable in advance. If one of the inserts could have been predicted to be less stable, it might have been the 28 nt antisense insert, which owing to its relative lack of structure might have been more accessible to endonuclease attack. Instead, the hammerhead ribozyme inserts consistently accumulated at 15- to 20-fold lower levels than any of the other inserts, whereas the antisense was, if anything, more stable than other inserts. We are unable to strictly interpret this observation. It is possible that this differential is caused by specific recognition and destruction of the hammerhead by some cellular activity, but whatever the cause it emphasizes the need to determine the levels of RNA in individual cases when evaluating efficacy of an insert. It also underscores one major reason to avoid, when possible, inclusion of unnecessary sequences that might be recognition sites for cellular proteins. The other reason is to avoid extra chances, however small, of potential toxicity when there is no compelling reason to have the extra sequence present.

The second unexpected conclusion was that the default pathway for all RNAs expressed from these promoters seems to be the nucleus. Although the partial RNA fusions were expected to be nuclear because they could not be recognized as processed tRNAs for transport, it was thought that the U6 transcripts might require a cap for nuclear retention. As shown in Figure 4, all of the U6 promoter transcripts gave primarily punctate nuclear staining whether or not the RNAs had 5′ caps. This suggests that if cytoplasmic localization is needed, it might be necessary to use active cytoplasmic localization signals.

The inability of ribozymes directed against the HIV U5 region to significantly inhibit HIV gene expression was unexpected, given the level of expression and the success reported in the past for ribozymes directed at this region.[20,30] This difference might be explained by the dif-

ferences in assay systems, although it is possible that RNAs expressed by these cassettes are simply unable to co-localize with the HIV RNA targets. The same arguments apply for the expression of TAR RNA. In contrast, expression of minimal Rev binding elements, both wild-type and a variety of artificially selected aptamers, are far more effective at inhibiting HIV gene expression in the proviral cotransfection. A large number of speculative hypotheses could be put forward to explain why Rev inhibitors work in this context while Tat inhibitors and the anti-RNA RNAs fail. For example, although both Rev and Tat have nuclear functions, the punctate locations of the expressed RNAs might make them unavailable to interact with those targets, but anti-Rev variants are specifically co-localized with Rev during some phase of its biosynthesis or transport.

Whatever the causes of the differential response to the various inserts, it is clear that it will be necessary to test hypothetical RNA therapeutics directly for effects on the intended targets in the context of different expression cassettes. It is hoped that once enough effective RNA inhibitor/expression cassette combinations can be identified, they can be delivered simultaneously to overcome mutational escape by the virus. The small size of both the promoter cassettes and the insert RNAs might make it possible to combine multiple cassettes in one vector. Nuclear-directed cassettes expressing several types of RNA inhibitors could be combined with cytoplasm-directed promoter cassettes for other types of RNAs.

Materials and methods

Preparation of recombinant clones

Construction of the U6+1, U6+19 and $tRNA^{met}$ expression cassettes was described previously.[36] Each of these cassettes was cloned into pTZ18U (Bio-Rad, Hercules, CA, USA) that had been restricted with *Bam*HI and *Hin*cII. The resulting vectors are pTZU6+1, pTZU6+19 and pTZtRNA. The U6+27 cassette was constructed by PCR amplification from 265 nucleotides upstream of the human U6 gene transcription initiation site through the first 27 nucleotides of the transcribed gene.[14,15,37] This fragment was substituted for the similar DNA segment of pTZU6+1. The *Sal*I cloning site was provided in the oligodeoxynucleotide used in the PCR.

The small RNAs expressed in the various expression vectors include two hammerhead ribozymes (HH1 and HH2), a hairpin ribozyme (hp),[20,30] two small RNA ligands for the Tat and Rev proteins (TAR RNA and RBE),[10,32,33,35] and HIV antisense RNA (AS). The inserts consisted of synthetic oligodeoxynucleotides, corresponding to the RNA sequences in Figure 2 plus flanking *Sal*I and *Xba*I cloning sites. The inserted sequences of the 'TAR' and 'RBE' aptamers are given in Figure 2 (RBE = 'Rev aptamer'). The other Rev aptamers tested for the data in Figure 5 were obtained by Giver *et al*.[33] Their sequences are:

RBE6 = 5'-GGGAACUCGAUGAAGCGAGCUCUUGGACACAGCCUCAAUGAGGCUGCAGAUACAAGUACUGACUUCGGAUC;
RBE63 = 5'-GGGAACTCGAUGAAGCGAAUUCUGGACUCCGUAUGCAAGUACGUUGAGCAACAGGCCUAUCUAUCGGAU;
RBE86 = 5'-GGGAACUCGAUGAAGCGAAUUCUGUGUAGAGGUACGCAAGUACGCGCUCCACAGGCCUAUCUAUCGGAU;
RBE126 = 5'-GGGAACUCGAUGAAGCGAGCUCUUGGACACAGCCUCAAUGAGGCUGCAGAAACAAGUACUGACUUCGGAUC.

Plasmid preparations were performed using the Plasmid Maxi Kit (Qiagen, Hilden, Germany) followed by a phenol:chloroform:isoamyl alcohol (24:24:1) extraction and ethanol precipitation. Sequences of expression cassettes and the cloned inserts were verified by DNA sequencing.

Transfections

Transfections of human 293 cells were carried out using a modified calcium phosphate protocol. The cells were split to 10–20% confluence in 7 ml DMEM (BioWhitaker, Walkersville, MD, USA), 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin on 100 mm plates prior to transfection. Five micrograms of plasmid were added to 0.5 ml of 244 mM $CaCl_2$, mixed dropwise into 0.5 ml 2 × HBS (270 mM NaCl, 1.5 mM Na_2HPO_4, 48 mM HEPES, pH 7.0), and placed dropwise evenly on the media, changing media daily. For the *in situ* visualizations the cells were plated to Petri dishes containing microscope cover slips that had been acid washed and coated with gelatin (type b: from bovine skin, 2% solution, Sigma, St Louis, MO, USA). Transfection efficiencies were tested by cotransfecting pCMVβ and then assaying the cells for β-galactosidase activity.[38]

Northern hybridizations

RNA samples prepared from transiently transfected human 293 cells, were separated on 6% acrylamide gels containing 8 M urea along with known RNA size markers.[39] The RNA was then blotted to maximum strength Nytran (Schleicher and Schuell, Keene, NH, USA) using the Genie electroblotter (Idea Scientific, Minneapolis, MN, USA) under conditions specified by the manufacturer. The RNA was UV-cross-linked to the Nytran by placing each side on a transilluminator (Fotodyne, Model 3–3000; New Berlin, WI, USA) for 2 min. Oligodeoxynucleotide probes to insert RNA were labeled using polynucleotide kinase (New England Biolabs, Beverly, MA, USA) and $\gamma^{32}P$ ATP. Separate probes were prepared against the hairpin ribozyme's catalytic domain, TAR and RBE transcripts and endogenous human U6 snRNA. The sequence of each is: 5'TACCAGGTAATATACCACAACGTGTGTTTCTCTG 3' (hairpin); 5' CTAGTCTAGACCAGAGAGCTCCCAGGCTCAGATCTGGGTCGAC - GCGT3' (TAR); 5'CTAGTCTAGAAGATACAGAGTCCACAAACGTGTTTCTCAATGCACCCGTCGACGCGT 3' (RBE); 5'GCGTGTCATCCTTGCGCAGGGGCC3' (U6 snRNA). RNA blots underwent a 1 h prehybridization at 60°C in 5 × SSPE buffer, 200 mg/ml salmon testis DNA (Sigma), 1% SDS. Hybridization was performed by adding labeled oligo probes to the prehybridization buffer and continuing incubation overnight. Blots were washed once with 50 ml of wash buffer (2 × SSPE, 1% SDS) for 10 min at room temperature, followed by three 100 ml washes, at the hybridization temperature, for 30 min each. The membrane was then exposed to a storage phosphor screen and subsequently scanned on the Molecular Dynamics PhosphorImager 445 SI (Sunnyvale, CA, USA). Image analysis, for quantification of hybridization

signals, was carried out using IP Lab Gel Software (Signal Analytic Corporation; Vienna, VA, USA). Expressed RNA copy number was estimated relative to endogenous U6 snRNA in each sample (400 000 copies per cell)[23] normalizing for approximate transfection efficiency measured by staining parallel cotransfections with pCMVβ.

Immunoprecipitations

The presence of a γ-phosphomethyl-GTP cap structure on transcripts was tested by immunoprecipitation essentially as described.[27] RNA (4 μg) prepared from transiently transfected human 293 cells was incubated with 40 μg of anti-me pppN cap antibody (provided by Ram Reddy, Baylor College of Medicine), for 40 min on ice, in NET2 buffer. Pansorbin (100 μl) (Calbiochem) was then added and allowed to incubate on ice for 40 min with occasional mixing. The bound portion was then pelleted by centrifugation, the supernatant was removed and saved. The pellet was then washed twice with NET2 buffer containing 0.05% NP40, the first of these two washes was pooled with the supernatant and then SDS was added to a concentration of 0.2%. The pellet containing bound RNA was then resuspended in NET2 buffer containing 0.2% SDS. Bound and unbound fractions were incubated at 65°C for 10 min and then extracted with equal volumes of phenol and then chloform. The RNA was then ethanol precipitated and analyzed by Northern hybridizations as above.

Labeling of in situ probes

Antisense probes were labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN, USA) by assymetric PCR using *Taq* DNA polymerase.[40] Synthetic oligodeoxynucleotides were constructed corresponding to the sense strands of human U6 RNA, hairpin ribozyme, TAR and Rev binding element (RBE) aptamer templates.[32] The probe sequences are: 5'GGAACGATAC AGAGAAGATTAGCATGGCCCCTGCGCAAGGATGA CACGCAAATTCGTGAAGCG3' (U6); 5'CCAGAGAAA CACACGTTGTGGTATATTACCTGGTACGCCGAATT CTAGAGCGGACTTCGGTCCGCTTT3' (hairpin); 5'CCA GATCTGAGCCTGGGAGCTCTCTGGTCTAGAGCGGA CTTCGGTCCGCTT3' (TAR); 5'GGGTGCAATGAGAAQ ACACGTTTGTGGACTCTGTATCTTCTAGAGCGGACT TCGGTCCGCTT3' (RBE). Priming oligodeoxynucleotides containing 17, 18, 21 and 21 nt were used that annealed to the 3' end of these templates. Labeling reactions typically contained: 10 mM Tris (pH 9.1), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 134 μM dTTP, 66 μM digoxigenin-11-dUTP, 2 units *Taq* DNA polymerase/100 μl reaction volume. Template concentrations ranged from 250 to 700 nmolar. The amount of priming oligo was either 20 or 50 times the amount of template oligo. Thirty cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s were performed. Single-stranded products were gel-isolated on 2% agarose gels. The digoxigenin-11-dUTP labeling efficiency of each probe was assayed using the Genius System Nonradioactive Nucleic Acid Detection Kit (Boehringer Mannheim).

In situ hybridizations

Human 293 cells were transfected as described above and after 36–48 h probed as described.[34] The final slide preparations were viewed using a Nikon (Garden City, NY, USA) Diaphot inverted microscope adapted for use with the Biorad Confocal Laser Scanning Imaging System (model MRC-600). Imaging was done with CoMOS Version 6.03 software (BioRad). Images shown in Figure 4b were obtained using the same settings to allow qualitative comparison of signal strengths.

HIV-1 antiviral assay

For determination of anti-HIV activity of the RNA aptamers, transient assays were performed by cotransfection of the expression plasmid DNA and infectious HIV proviral DNA. 293 Cells were grown for 24 h in six-well polystyrene tissue culture plates in 2 ml EMEM supplemented with 10% FBS, and transfected according to a modified calcium phosphate transfection method of Wigler *et al*.[41] The DNA mixture consisted of 4 μg candidate or control test plasmid, 0.1 μg human growth hormone (HGH) transfection control pTKGH (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA), and 0.1 μg HIV-1 DNA pNL4–3 (NIH AIDS Research and Reference Reagent Program, Bethesda, MD, USA). After 5 h, the DNA precipitate medium was replaced with fresh EMEM-10% FBS and observed for 3 days. Supernatant fluid was collected and analyzed for reverse transcriptase (RTase) activity as described.[42] ^{32}P-TTP incorporation was measured using a phosphoimager (Ambis, San Diego, CA, USA). Human growth hormone was measured using an enzyme immunoassay (HGH-GES, Nichols Institute Diagnostics) and did not influence the growth of HIV-1. RTase results were used only if the transfection control showed the presence of HGH.

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